RECOMBINANT DNA PREPARED BY INTEGRATING DNA THAT CODES FOR MYOSIN HEAVY-CHAIN SM1 ISOFORM PROTEIN INTO VECTOR DNA, AND MICROORGANISM AND ARTERIOSCLEROSIS REMEDY BOTH CONTAINING THE RECOMBINANT DNA

Patent number: WO9623069

Publication date: 1996-08-01

Inventor: HASEGAWA KAZUHIDE (JP); TAKAHASHI KATSUHITO (JP); ARAKAWA EMI (JP); ODA SHOJI (JP); ISHIYAMA HARUO (JP); MATSUDA YUZURU (JP); SUGAWARA MICHIHIRO (JP)

Applicant: HASEGAWA KAZUHIDE (JP); TAKAHASHI KATSUHITO (JP); ARAKAWA EMI (JP); ODA SHOJI (JP); ISHIYAMA HARUO (JP); MATSUDA YUZURU (JP); OSAKA PREFECTURE (JP); SUGAWARA MICHIHIRO (JP); VESSEL RESEARCH LAB CO LTD (JP)

Classification:

- international: C12N15/12; C12N1/21; A61K38/17

- european: C07K14/47A7

Application number: WO1996JP00134 19960125

Priority number(s): JP19950010085 19950125

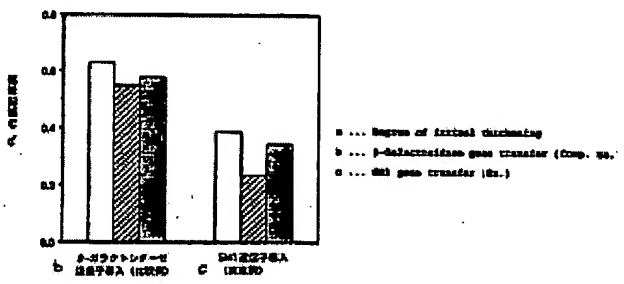
Also published as:

EP0806477 (A1)
US6593304 (B1)
FI973104 (A)
EP0806477 (A4)
EP0806477 (B1)

more >>

Abstract of **WO9623069**

A recombinant DNA prepared by integrating a DNA that codes for a smooth-muscle myosin heavy-chain SM1 isoform protein into a vector DNA; and a microorganism and arteriosclerosis remedy both containing the recombinant DNA. The DNA is utilizable as a gene remedy for post-PTCA reconstriction.



RECOMBINANT DNA PREPARED BY INTEGRATING DNA THAT CODES FOR MYOSIN HEAVY-CHAIN SM1 ISOFORM PROTEIN INTO VECTOR DNA, AND MICROORGANISM AND ARTERIOSCLEROSIS REMEDY BOTH CONTAINING THE RECOMBINANT DNA

Description of correspondent: **EP0806477**

TECHNICAL FIELD

The present invention relates to recombinant DNA comprising DNA coding for myosin heavy chain SM1 isoform protein inserted into vector DNA, a microorganism carrying the recombinant DNA and an agent for treatment of arteriosclerosis comprising the recombinant DNA which are used in gene therapy.

BACKGROUND ART

Smooth muscle-type myosin heavy chain SM1 isoform protein is responsible for contraction and relaxation of smooth muscles, and is one of the myosin heavy chain isoform proteins expressed specifically in smooth muscle cells. As the DNA coding for the protein, the nucleotide sequence of cDNA coding for rabbit SM1 isoform is known (P. Babij et al.: Proc. Natl. Acad. Sci. USA, 88, 10676 (1991)), but there is not known any homology among nucleotide sequences for such DNAs. Further, there is not known any recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA which recombinant DNA can be injected into animal cells.

It has been reported that when cDNA coding for a protein called calponin which is a protein existing in smooth muscle cells is introduced and expressed in a vascular smooth muscle cell line derived from rat pulmonary arteries, the time required for doubling the cells is prolonged by about 4 hours (Takahashi et al.: Circulation, 88, I-174 (1993)) and that when cDNA for human calponin is injected into a rabbit topically at a site where the carotid artery has been abraded with a balloon, thickening of the intima is inhibited (Takahashi et al.: Circulation, 88, I-656 (1993)). It is not known that recombinant DNA comprising DNA coding for myosin heavy chain SM1 isoform protein inserted into vector DNA has pharmacological effect and is used for in gene therapy.

For treatment of arteriosclerosis, percutaneous transluminal coronary angioplasty (PTCA) in which a stenosed site is enlarged by a balloon catheter is extensively conducted. The treatment method has the advantages of easier operation and higher degree of success than the bypass surgery etc., while the treatment method has the disadvantage that 30 to 40 % of the patients after the treatment suffer from restenosis of blood vessels due to abnormal proliferation of vascular cells. Therefore, there is demand for developments in an agent for treatment of arteriosclerosis effectively used for preventing such restenosis.

DISCLOSURE OF THE INVENTION

The present invention includes:

(1) A recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA.
(2) The recombinant DNA according to (1) wherein the vector DNA is a retrovirus vector, adenovirus vector, adeno-associated virus vector or a plasmid capable of being expressed in an animal.
(3) The recombinant DNA according to (2) wherein the plasmid capable of being expressed in an animal is pCXN2 or PAGE208.
(4) The recombinant DNA according to any one of (1) to (3) wherein the smooth muscle-type myosin heavy chain SM1 isoform protein is of a human smooth muscle type, rabbit muscle type or mouse muscle type.
(5) The recombinant DNA according to any one of (1) to (3) wherein the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein is the nucleotide sequence shown in SEQ ID NO::1, or the nucleotide sequence shown in SEQ ID NO:1 in which at least one nucleotide is added, deleted or replaced.
(6) DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein, which is the nucleotide

sequence shown in SEQ ID NO:1, or the nucleotide sequence shown in SEQ ID NO:1 in which at least one nucleotide is added, deleted or replaced.
(7) A microorganism carrying the recombinant DNA of (5).
(8) The microorganism according to (7) which belongs to the genus Escherichia.
(9) An agent for treatment of arteriosclerosis which comprises the recombinant DNA of any one of (1) to (5).

The DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein includes cDNA or genomic DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein, preferably DNA derived from humans, rabbits, mice, etc.

The nucleotide sequence of the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein includes the nucleotide sequence of cDNA coding for rabbit SM1 isoform protein (Proc. Natl. Acad. Sci. USA, 88, 10676 (1991)), the nucleotide sequence of the DNA coding for mouse myosin heavy chain SM1 isoform protein as shown in SEQ ID NO:1, and the nucleotide sequence as shown in SEQ ID No:2 which is specified by homologous regions between the above nucleotide sequences (In the Sequence Listing, Y represents T, U or C; S represents G or C; V represents A, G or C; B represents G, C, T or U; W represents A, T or U; N represents A, C, G, T, U or a single bond; and the codon YAS (388-390) represents TAC or CAG.).Accordingly, the nucleotide sequence shown in SEQ ID NO:2 contains the nucleotide sequence of SEQ ID NO:1 and the nucleotide sequence of the cDNA coding for rabbit SM1 isoform protein. A partial nucleotide sequence of cDNA coding for human smooth muscle-type myosin heavy chain SM1 isoform protein is also known (Amer. J. of Medical Genetics, 46, 61-67 (1993)), and a nucleotide sequence of this partial nucleotide sequence combined with a partial nucleotide sequence of SEQ ID NO:2 is also contained in the nucleotide sequence of the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein according to the present invention.

Further, the nucleotide sequence of SEQ ID NO:2 or the combination of a partial nucleotide sequence of SEQ ID NO:2 and the nucleotide sequence of the DNA derived from human smooth muscles, in which one or more nucleotides have been added, deleted or replaced by means of site-directed mutagenesis (Nucleic Acid Research, 10, 6487-6508 (1982)), is also contained in the nucleotide sequence of the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein according to the present invention.

The vector DNA that can be used includes virus vectors such as retrovirus vector, adenovirus vector and adeno-associated virus vector and plasmids capable of being expressed in an animal such as pCXN2 (Gene, 108, 193-200 (1991)) and PAGE207 (Japanese Patent Laid-Open Publication No. 46841/1994) as well as their modified vectors.

The agent for treatment of arteriosclerosis according to the present invention can be produced by compounding the recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA as an active ingredient together with a base used in agents for gene therapy. Just before administration, the pharmaceutical preparation can be used in gene therapy for arteriosclerosis, if necessary after encapsulation in liposomes etc. (Proc. Natl. Acad. Sci. USA., 90, 11307 (1993)).

Where the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein is inserted into a virus vector, a therapeutic agent can be produced by preparing virus particles containing the recombinant DNA and then compounding them together with a base used in agents for gene therapy (Nature Genet., 8, 42(1994)).

The base used in agents for gene therapy may be any base generally used in injections. The base includes, for example, distilled water, a salt solution of sodium chloride, a mixture of sodium chloride and an inorganic salt, or the like, solutions of mannitol, lactose, dextran, glucose, etc., solutions of amino acid such as glycine, arginine, etc., a mixed solution of an organic acid solution or a salt solution and glucose solution, and the like. Further, injections may be prepared in a usual manner as a solution, suspension or dispersion by adding adjuvant such as an osmotic pressure controlling agent, pH adjusting agent, vegetable oils such as sesame oil, soybean oil, etc. or surface active agents such as lecithin, non-ionic surface active agent, etc. to the above base. These injections can also be powdered, lyophilized, etc. to be dissolved just before use.

The agent for treatment of arteriosclerosis can be used as such in the case of a solution, and in the case of a solid it is dissolved in the base previously sterilized if necessary just before use in gene therapy.

The method for administration of the agent for treatment of arteriosclerosis according to the present invention involves topically administrating it into a patients who underwent the PTCA treatment, at a dose

of 1 ng to 1 g per day or once after the surgery by means of a catheter etc. such that it can be absorbed into their vascular smooth muscle cells at the target site.

The agent for treatment of arteriosclerosis according to the present invention is safe in this dose range.

Hereinafter, the process for producing the recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA is described.

The recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA can be obtained according to the usual genetic engineering method described below or its modified method.

A DNA clone coding for smooth muscle-type myosin heavy chain SM1 isoform protein is detected in a cDNA library from tissues composed mainly of smooth muscles in the uterus, aorta, etc., according to the method described by A. Abe et al. (ECL direct DNA labeling detection system manual (Amersham)).

The recombinant DNA comprising the inserted DNA coding for myosin heavy chain SM1 isoform protein can be produced by ligating said DNA fragment to a downstream region from a promoter in a suitable vector DNA (J. Sambrook et al., Molecular Cloning, 2nd Ed., Vol. 1, Cold Spring Harbor Laboratory Press (1989)). Animal cells are used as a host, and a promoter derived from SV40, a promoter from retrovirus, a metallothionein promoter, beta -actin promoter etc. can be utilized as the promoter. For the expression, the use of an enhancer is also effective.

The reaction conditions for the above-mentioned recombinant techniques are as follows: The digestion of DNA with a restriction enzyme is carried out by allowing the restriction enzyme in an amount of 0.1 to 100 units, preferably 1 to 3 units per mu g DNA to act on 0.1 to 20 mu g DNA in a reaction solution usually containing 2 to 200 mM preferably 10 to 40 mM Tris-HCl buffer, pH 6.0 to 9.5, preferably pH 7.0 to 8.0, 0 to 200 mM sodium chloride, and 2 to 20 mM preferably 5 to 10 mM magnesium chloride, at 20 to 70 DEG C (the optimum temperature is varied depending on the restriction enzyme used) for 15 minutes to 24 hours. The termination of the reaction can be effected usually by heating the reaction solution at 55 to 75 DEG C for 5 to 30 minutes or by inactivating the restriction enzyme with reagents such as phenol etc.The DNA fragment, or the gapped duplex DNA, generated by digestion with the restriction enzyme can be purified using Prep-A-Gene Matrix (Bio-Rad). The ligation of the DNA fragment can be effected using a DNA ligation kit (Takara Shuzo Co., Ltd.).

The recombinant DNA containing DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein thus constructed in this manner is used to produce a transformant.

The host for the plasmid capable of being expressed in an animal includes, for example, microorganisms belonging to the genus Escherichia, such as Escherichia coli K12 . HB101 (H. W. Boyer et al.: J. Mol. Biol., 41, 459 (1969)), DH5 alpha (D. Hanahan: J. Mol. Biol., 166, 557 (1983)) etc. The transformation of microorganisms of the genus Escherichia can be effected according to the method of Cohen et al. (S. N. Cohen et al.: Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)).

The host used for the virus vector includes animal cells having the ability to produce viruses, such as monkey cell COS-7, Chinese hamster cell CHO, mouse cell BALB/3T3, human cell HeLa, etc.; the host for the retrovirus vector includes PSI CRE, PSI CRIP (Proc. Natl. Acad. Sci. USA, 85, 6460 (1988)), MLV (J. Virol., 65, 1202 (1991)) etc.; and the host for the adenovirus vector and adeno-associated virus vector includes 293 cells derived from human fetal kidney ("Jikken Igaku" (Experimental Medicine), 12, 316 (1994)) etc. The introduction of the virus vector into animal cells can be effected using the calcium phosphate method (Virology, 52, 456 (1973)) etc.

The resulting transformant can be cultured in the following manner depending on the difference in the cell species to produce the recombinant DNA.

To culture the transformant from a microorganism of the genus Escherichia as the host, the suitable medium is a liquid medium containing a carbon source, nitrogen source, inorganic substance, etc. necessary for the growth of the transformant. The carbon source includes e.g. glucose, dextrin, soluble starch, sucrose, glycerol, etc. ; the nitrogen source includes e.g. an ammonium salt, peptone, casein, etc.; and the inorganic substance includes e.g. calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc. A yeast extract, vitamins, etc. may further be added. The pH of the medium is preferably about 5 to 8. To culture the microorganism of the genus Escherichia, the medium is preferably Terrific broth (K. D. Tartof et al.: Bethesda Res. Lab. Focus, 9, 12 (1987)) or the like.The transformant is cultured usually at about 15 to 43 DEG C for about 8 to 24 hours, if necessary under aeration or stirring. After the culture is finished, the recombinant DNA containing DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein can be obtained through purification by the Birnboim method (Nucleic Acid

Res., 7, 1513 (1979)), etc.

To culture the transformant from animal cells as the host, the medium used includes a medium containing about 5 to 20 % fetal bovine serum, such as 199 medium (Morgan et al.: Proc. Soci. Biol. Med., 73, 1 (1950)), MEM medium (H. Eagle: Science, 122, 501 (1952)), DMEM (R. Dulbecco et al.: Virology, 8, 396 (1959)) or the like. The pH is preferably in the range of about 6 to 8. The transformant is cultured usually at about 30 to 40 DEG C for about 18 to 60 hours, if necessary under aeration or stirring.

Because virus particles containing the recombinant DNA are released into the culture supernatant, the recombinant DNA containing DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein can be obtained from the supernatant by concentrating and purifying virus particles by the cesium chloride centrifugation method (Koji Sawada et al.: "Shin Seikagaku Jikken Kouza 2-V" (New Biochemistry Experimental Course 2-V), 33 (1992)), the polyethylene glycol precipitation method (Arch. Virol., 71, 185 (1982)), the filter concentration method (J. Cell. Biol., 111, 217 (1990)), etc.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a restriction enzyme map of the cDNA coding for mouse smooth muscle-type myosin heavy chain SM1 isoform protein and the location of the coding region.

Plasmid name: SM-1 F text
Plasmid size: 6175 bp
Region in the frame: coding region
A: ApaI
B: BamHI
Sa: SacI
Sm: SmaI

FIG. 2 shows the area of CHO cell colonies selected with hygromycin after introduction of plasmid pSE-SM1-Hyg vs. the area of CHO cell colonies after introduction of plasmid PAGE208.

FIG. 3 shows the area of HeLa cell colonies selected with hygromycin after introduction of plasmid pSE-SM1-Hyg vs. the area of HeLa cell colonies after introduction of plasmid PAGE208.

FIG. 4 shows the comparison among the proliferation rates of the wild type, the PAGE208-introduced clone and the SM1 isoform-expressing clones (SM1-5-2-1, SM1-5-3-3) of CHO cells by the MTT method.

- &cir& - CHO
- INCREMENT - PAGE208-1
- &cirf& - SM1 5-2-1
- &squf& - SM1 5-3-3

FIG. 5 shows the degrees of thickening of the intima after introduction of the SM1 gene (Example) and beta -galactosidase gene (Comparative Example) into the vessel walls in a balloon injured rabbit model.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in more detail with reference to Examples which however are not intended to limit the scope of the present invention.

Example 1. Recombinant DNA comprising DNA coding for mouse smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA

(a) Preparation of a probe

Plasmids pSMHC29 and pIH61 in which a cDNA fragment coding for rabbit smooth muscle-type myosin

heavy chain SM2 isoform protein had been cloned (J. Biol. Chem., 264, 9734-9737 (1989)) were used. These were digested with EcoRI (a product of Takara Shuzo Co., Ltd.; the restriction enzymes used hereinafter are products of Takara Shuzo Co., Ltd. unless otherwise specified) to prepare a cDNA fragment of rabbit smooth muscle-type myosin heavy chain SM2 isoform protein to be inserted. The cDNA fragment was labeled using an ECL direct DNA labeling system (Amersham) to be used as a probe in the following screening.

(b) Screening

A lambda gt11 vector mouse uterus cDNA library (Clontech) was mixed with E. coli Y1090 (Clontech) and incubated at 37 DEG C for 15 minutes, followed by adding 0.7 % agarose NZY (Nucleic Acid Res., 16, 7583-7600 (1988)), and it was then spread on a 1.5 % agar NZY plate. A nylon filter was placed on the plate wherein plaques occurred so that the plaques were transferred to the filter. This filter was subjected to denaturation by alkali treatment and the DNA was fixed by heating at 80 DEG C for 2 hours. The DNA was hybridized to the probe previously labeled according to the protocol of the ECL direct DNA labeling system in (a) above. Then, the hybridized clones were detected by autoradiography using an ECL detection system (Amersham).

(c) Analysis of DNA nucleotide sequence

Four clones were identified and each clone was subcloned in pUC119 (Takara Shuzo Co., Ltd.) or pBluescript SK(-) (Stratagene) for analysis of the DNA nucleotide sequence. E. coli DH5 alpha was transformed with the resulting plasmids to give transformants Escherichia coli DH5 alpha /pmsmhc20, pmsmhcC5, pmsmhcN08 and pmsmhcN14. The plasmids produced by the transformants were subjected to stepwise deletion by digestion with an exonuclease or to digestion with a suitable restriction enzyme, followed by self-cyclization or subcloning to prepare template DNA for sequence analysis (Gene, 28, 351-359 (1984)). A fluorescence type DNA sequencer (Applied Biosystems) was used for sequencing and MacMolly (Soft Gene GmbH) was used for data analysis. The nucleotide sequence thus determined is shown in SEQ ID NO:1. The amino acid sequence deduced from the nucleotide sequence is also shown in SEQ ID NO:1.

(d) Construction of an expression plasmid for mouse smooth muscle-type myosin heavy chain SM1 isoform

From the 4 clones previously obtained, plasmid pmSM1 containing cDNA coding for the whole coding region for SM1 isoform was prepared in the following manner (J. Sambrook et al:, Molecular Cloning, 2nd Ed., Vol. 1, Cold Spring Harbor Laboratory Press, 1989). To shorten the 5'-non-coding region, plasmid pmsmhcN14 i.e. one of the 4 clones was digested with restriction enzymes BamHI and Bg1II and a 5-kb DNA fragment was purified and recovered therefrom. The DNA fragment was self-cyclized and transformed into E. coli DH5 alpha to give an ampicillin resistant colony. From the colony, a desired microorganism is picked up and cultured, and plasmid DNA was recovered using a known method (H. C. Birnboim et al.: Nucleic Acid Res., 7, 1513 (1979)). The structure of the plasmid thus obtained was confirmed by agarose gel electrophoresis after digestion with BamHI and KpnI.The plasmid was designated pmsmhcN14'.

The resulting plasmid pmsmhcN14' was digested with restriction enzymes NsiI (New England Biolabs) and EcoRV, and a 4-kb DNA fragment was purified and recovered therefrom. Separately, plasmid pmsmhcN08 was also digested with NsiI and EcoRV, and a 1-kb DNA fragment was purified, recovered and ligated into the previously obtained 4-kb DNA fragment derived from plasmid pmsmhcN14', whereby plasmid pmsmhcN14-08 was constructed. The structure of this plasmid was confirmed by digestion with NsiI and EcoRV. The resulting plasmid pmsmhcN14-08 was digested with restriction enzymes ApaI and KpnI and a 5-kb DNA fragment was purified and recovered therefrom. Plasmid pmsmhc20 was also digested in a similar manner with ApaI and KpnI, and a 1.5-kb DNA fragment was purified, recovered and ligated into the previously obtained 5-kb DNA fragment, whereby plasmid pmsmhcN14-08-20 was constructed.The structure of the plasmid was confirmed by digestion with ApaI and KpnI.

The resulting plasmid pmsmhcN14-08-20 was digested with restriction enzyme ApaI and a 6-kb DNA fragment was purified and recovered therefrom. Plasmid pmsmhcC5 was also digested with ApaI, and a 2-kb DNA fragment was purified, recovered and ligated into the 6-kb DNA fragment from pmsmhcN14-08-20 whereby plasmid pmsmhcN14-08-5-20 was constructed. The structure of this plasmid was confirmed by

digestion with EcoRI and NruI.

Plasmid pmsmhcN14-08-5-20 was digested with restriction enzymes NsiI and NruI and a 8-kb DNA fragment was purified and recovered therefrom. Plasmid pmsmhcN08 was also digested in a similar manner with NsiI and NruI, and a 1.5-kb DNA fragment was purified, recovered and ligated into the previously obtained 8-kb DNA fragment, whereby plasmid pmSM1 containing the whole coding region for mouse smooth muscle-type myosin heavy chain SM1 isoform was constructed. The structure of this plasmid was confirmed by digestion with NsiI and NruI.

The resulting plasmid pmSM1 was digested with restriction enzymes XbaI and KpnI and a 6-kb DNA fragment was purified and recovered therefrom. Separately, expression vector PAGE208 derived from expression vector PAGE207 (Japanese Patent Laid-Open Publication No. 46841/1994) by deleting an SmaI site located in a promoter region for hygromycin B resistance gene in PAGE207 was digested with XbaI and KpnI, and a 6-kb DNA fragment was purified, recovered, and ligated into the previously obtained 6-kb DNA fragment derived from plasmid pmSM1, whereby expression plasmid pSE-SM1-Hyg was constructed. The structure of the plasmid was confirmed by digestion with XbaI and KpnI.

A DNA fragment obtained by digestion of plasmid pmSM1 with XbaI and KpnI was blunt-ended with a DNA blunting kit (Takara Shuzo Co., Ltd.). Separately, expression vector pCXN2 was digested with XhoI and blunt-ended with the DNA blunting kit (Takara Shuzo Co., Ltd.). These 2 kinds of DNA were ligated to construct expression plasmid pCAG-SM1. The structure of the plasmid was confirmed by digestion with Bg1II.

The above plasmid pSE-SM1-Hyg was inserted into Escherichia coli whereby a transformant was obtained.

The transformant has been deposited as Escherichia coli pSE-SM1-Hyg (FERM BP-4974) with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan, since January 24, 1995.

Example 2. Effect of expression of SM1 isoform in CHO cells and HeLa cells on cell proliferation

2 mu g of expression plasmid pSE-SM1-Hyg and 4 mu l of lipofectamine<(TM)> (Gibco) were mixed and incubated for 15 minutes to produce a complex. The complex was added to CHO cells or HeLa cells and incubated at 37 DEG C for 5 hours so that the gene was introduced into the cells. The cells were diluted and spread on a vessel, and cells into which the gene had been introduced were selected in the presence of hygromycine<(TM)> (Sigma). About 10 days later, the drug resistant cell colonies were stained with Coomassie Blue<(TM)> (Bio-Rad) and their area was measured with an image analyzer. As a result, many of the colonies had smaller areas than those of the colonies carrying the introduced control vector PAGE208, indicating that their proliferation was inhibited by the expression of the SM1 isoform (FIGS. 2 and 3).Separately, the hygromycin resistant CHO cells were cloned and the resulting clones SM1-5-2-1 and SM1-5-3-3 were examined for their proliferation rate. The result indicated that their proliferation rate was lower than that of the wild-type or PAGE208-introduced cells (FIG. 4).

Example 3. Inhibitory effect of forcible introduction of SM1 gene into vessel walls in a balloon injured rabbit model on thickening of the intima

A PTCA catheter at 3 French (Fr) was inserted under X-raying through the femoral artery into the right common carotid artery of a Japanese white domestic rabbit weighing 2.4 to 3.2 kg. The right common carotid artery was abraded 3 times with a balloon expanded at 10 atm. to injure the vessel wall. Three days after the abrasion, the injured portion of the vessel was infused with a mixture of 300 mg pCAG-SM1 and lipofectin (Gibco BRL) through a Wolinsky type infusion catheter (a product of BARD) at 6 atm.. For Comparative Example, other domestic rabbits were treated in the same manner, and in place of pCAG-SM1. an expression vector for beta -galactosidase was introduced into the injured portion of the vessel.

Three days after the infusion, the animals were sacrificed by perfusion with heparin physiological saline and their right common carotid arteries were removed and cut thin, followed by adding 1 ml ISOGEN (Wako Pure Chemical Industries, Ltd.), and the tissues were homogenized with a Polytron homogenizer. The whole RNA was extracted by the AGPC method (P. Chomczynski and N. Sacchi: Anal. Biochem., 162, 156-159 (1987)), and the mRNA for SM1 was amplified by the RT-PCR method. The amplified DNAs from the Example (introduction of SM1 gene) and Comparative Example (introduction of beta -galactosidase gene) and their fragments digested with restriction enzyme NheI were subjected to agarose

gel electrophoresis. The SM1 band was detected strongly in the Example and faintly in the Comparative Example, and the band in only the Example was digested with NheI.The supported that the SM1 gene derived from the mouse was expressed in the Example because the digestion site of the restriction enzyme is not present in the DNA for SM1 derived from the domestic rabbit but present in the mouse SM1.

Two weeks after introduction of the gene, the rabbits were sacrificed by perfusion with PBS containing 2 % paraformaldehyde and fixed, and their right common carotid arteries were removed. The part into which the infusion catheter had been introduced was dissected out and embedded in paraffin, and a thin round section of the vessel was prepared. One vessel was divided equally into 4 tissues and 1 section was prepared from each tissue and stained with hematoxylineosin. The vessel was divided along the internal elastic plate as the boundary into the intima and media, and the area of each was determined. The intima area/media area ratio was determined for each section and the mean was determined. The degree of thickening of the intima was determined using the mean.From the degree of thickening of the intima as determined for 3 animals in the Example and 3 animals in the Comparative Example, it was confirmed that thickening of the intima was inhibited by forcible introduction of the SM1 gene (FIG. 5).

The results suggested that plasmids pSE-SM1-Hyg and pCAG-SM1 possess the effect of depressing cell proliferation and are effective in restenosis of arteriosclerosis, caused by abnormal proliferation of vascular cells.

INDUSTRIAL APPLICABILITY

It was revealed that the proliferation of animal cells is inhibited when the recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA according to the present invention is expressed in the animal cells. The effect was also observed in human-derived HeLa cells. Therefore, the recombinant DNA of the present invention can be used effectively as an agent for gene therapy of restenosis after PTCA treatment.

EMI18.1

EMI19.1

EMI20.1

EMI21.1

EMI22.1

EMI23.1

EMI24.1

EMI25.1

EMI26.1

EMI27.1

EMI28.1

EMI29.1

EMI30.1

EMI31.1

EMI32.1

EMI33.1

EMI34.1

EMI35.1

RECOMBINANT DNA PREPARED BY INTEGRATING DNA THAT CODES FOR MYOSIN HEAVY-CHAIN SM1 ISOFORM PROTEIN INTO VECTOR DNA, AND MICROORGANISM AND ARTERIOSCLEROSIS REMEDY BOTH CONTAINING THE RECOMBINANT DNA

Claims of correspondent: **EP0806477**

1. A recombinant DNA comprising DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein inserted into vector DNA.

2. The recombinant DNA according to claim 1 wherein the vector DNA is a retrovirus vector, adenovirus vector, adeno-associated virus vector or a plasmid capable of being expressed in an animal.

3. The recombinant DNA according to claim 2 wherein the plasmid capable of being expressed in an animal is pCXN2 or PAGE208.

4. The recombinant DNA according to claim 1 wherein the smooth muscle-type myosin heavy chain SM1 isoform protein is of a human smooth muscle type, rabbit smooth muscle type or mouse smooth muscle type.

5. The recombinant DNA according to claim 1 wherein the DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein is the nucleotide sequence shown in SEQ ID NO:1, or the nucleotide sequence shown in SEQ ID NO:1 in which at least one nucleotide is added, deleted or replaced.

6. DNA coding for smooth muscle-type myosin heavy chain SM1 isoform protein, which is the nucleotide sequence shown in SEQ ID NO:1, or the nucleotide sequence shown in SEQ ID NO:1 in which at least one nucleotide is added, deleted or replaced.

7. A microorganism carrying the recombinant DNA of claim 5.

8. The microorganism according to claim 7 which belongs to the genus Escherichia.

9. An agent for treatment of arteriosclerosis which comprises the recombinant DNA of claim 1.
